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(54) Title: METHOD FOR DETECTION OF ANTIGENS OF A RETROVIRUS ASSOCIATED WITH AIDS IN SERUM AND OTHER BODY FLUIDS

(57) Abstract

A rapid and sensitive assay for the detection of antigens of a human retrovirus indicative of acquired immune deficiency syndrome (AIDS) or AIDS related complexes (ARC) by forming and detecting triple sandwich immune complexes, and diagnostic test kits for carrying out the method.

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METHOD FOR DETECTION OF ANTIGENS OF A RETROVIRUS ASSOCIATED WITH AIDS IN SERUM AND OTHER BODY FLUIDS

TECHNICAL FIELD

The present invention relates to a sensitive assay method for detecting the presence or absence of antigens of a human retrovirus indicative of Acquired Immune Deficiency Syndrome (AIDS), and AIDS-related complexes (ARC) in serum, plasma and other body fluids and in cell lysates, particularly, lysates of leukocytes or a subpopulation thereof. The present invention also relates to test kits for carrying out the assay method.

BACKGROUND ART

AIDS was first diagnosed in approximately 1981, but the causative agent was not yet known (as used herein the term AIDS refers to AIDS and ARC). Intensive research efforts resulted in identification of a retrovirus identified as HTLV-III/LAV, now called HIV-1, as one of the etiologic agents of AIDS. It has been recently reported that another human retrovirus, designated HIV-2 also causes AIDS.

The human retroviruses which are associated with AIDS are believed to be transmitted through intimate sexual contact as well as through blood. Infections caused by such human retroviruses result in the appearance of antibodies in the serum, semen and other body fluids of infected victims. The antibodies are generally directed against certain viral core and envelope proteins including the HIV-1 core proteins designated p 24 (24,000 MW), and envelope proteins designated gp 41 (41,000 MW), gp 120 (120,000 MW) and

gp 160 (160,00 MW). The core and envelope proteins have been identified by, among others, Western Blot techniques.

The AIDS viruses cripple the body's immune defense system. The viruses target T_4 lymphocytes, in particular a T-lymphocyte subset known as helper lymphocytes. Infection with any one of the AIDS viruses results in reduction in the number and a change in the function of T_4 cells and/or an increase in T_8 subsets; other T-lymphocytes as well as B-lymphocytes are eventually also affected by the virus, resulting in collapse of the immune system.

Presently, it is believed that homosexual men, bisexual men and abusers of injected drugs are at the highest risk of infection. Recipients of blood products and organ transplantations are also at risk of contracting AIDS.

There are several known methods for detecting antibodies to human retroviruses associated with AIDS, including, the enzyme-linked immunosorbance assay (ELISA), Western Blot, Quick Western Blot (copending applications Serial Nos. 871,505 filed June 6, 1986 and 99,311 filed September 21, 1987), and immunofluorescence techniques. Sarngadahran, M. G., et al, Science 224:506, 1983; Tsang et al, Methods of Enzymology, 92:29, 1983 Academic Press; Safai, B. et al, Lancet, 1:1438, 1984; and Essex, M. et al, Science 220:859, 1983.

The established screening process, which involves detecting the presence of antibodies to the AIDS viruses, is not entirely satisfactory because detectable amounts of such antibodies are not produced until at least two to four months after primary infection. Therefore, early diagnosis of AIDS by the detection of antibodies during this so-called "gray area" is not possible. As can be appreciated a technique for detecting retroviral antigens, rather

than antibodies, in patients suspected of infection is a useful tool for early detection of AIDS. Early diagnosis facilitates treatment, and minimizes the risk of spreading the infection.

An "HTLV-III Capture Assay" developed by Integrative Diagnostics, Inc., 1961 Sulphur Spring Road, Baltimore, MD 21227, is designed to detect products of viral replication with a sandwich ELISA system. The assay, however, does not detect viral antigens directly from serum or cells. Rather, the test requires obtaining lymphocytes suspected of carrying the retrovirus and culturing these lymphocytes with disease free cells from a healthy donor for about one to two weeks. Only then can an ELISA test be performed to determine whether viral antigens are present. Thus, the HTLV-III Capture Assay is impractical for mass screening because it is both time consuming, expensive and does not detect antigens directly from a test sample obtained from a patient.

The present assay technique provides a method for detecting picogram amounts of retroviral antigens directly from serum, body fluids and lysed cell materials.

DISCLOSURE OF THE INVENTION

The present invention provides a rapid and sensitive test system for detecting the presence of antigens of a human retrovirus indicative of AIDS or ARC, by forming a triple sandwich immune complex comprising antibody-antigen-antibody anti-antibody. The method of the invention comprises incubating antibodies to a human retrovirus indicative of AIDS with a biological fluid for a time sufficient for the retroviral antibodies and antigens in the biological fluid to form a first immune complex comprising antibody-antigen; the first immune complex is then incubated with antibodies to the retrovirus for a time sufficient for the retroviral antibodies and first

immune complex to form a second immune complex comprising antibody-antigen-antibody; the second immune complex is then incubated with retroviral anti-antibodies for a time sufficient to allow formation of a third immune complex comprising antibody-antigen-antibody-anti-antibody and detecting the formation of the third immune complex.

The assay method of the invention may be carried out by either a modified ELISA (enzyme linked immunosorbance assay) or semi-solid phase enzyme-linked dot spot test. The dot spot test preferably employs nitrocellulose paper, typically cut into strips.

In accordance with the present method, picogram amounts of AIDS retroviral antigens are detectable directly from body fluids or lysed cell material. Results are obtainable in about 19 hours, less than 1/15 the time required for the conventional HTLV-III Capture Assay.

In one embodiment of the present invention, a diagnostic test kit is provided which permits on site testing for retroviral antigens. The kit comprises positive and negative reference controls, washing buffers, antibody reagents, nitrocellulose strips and enzyme conjugates necessary to carry out the method of the invention on test samples.

The diagnostic kit also preferably includes predeveloped positive and negative reference strips and reagent control strips for evaluating the test results by visual comparison with the test strips. The inclusion of predeveloped strips facilitates reading the test results and virtually eliminates the need for trained personnel. This is especially important for on site testing where the facility may not have specialized equipment required for other testing systems.

MODES FOR CARRYING OUT THE INVENTION

The semi-solid assay method of the invention comprises forming and detecting triple sandwich immune

complexes comprising antibody - antigen - antibody - anti-antibody; the triple sandwich immune complexes are formed by (1) incubating antibodies to a retrovirus indicative of AIDS with a biological fluid or lysed cell material to obtain a first immune complex comprising antibody - antigen; (2) incubating the first immune complex with antibodies to a retrovirus indicative of AIDS to obtain a second immune complex comprising antibody - antigen - antibody and (3) incubating the second immune complex with an anti-antibody against the antibody of step (2) to obtain a triple sandwich immune complex comprising antibody - antigen - antibody - anti-antibody.

Antibodies To A Retrovirus Indicative Of Aids

In accordance with the method of the invention, antibodies to a retrovirus indicative of AIDS are coated onto a solid phase, such as microtiter plates, round plastic or glass beads, approximately 0.5-2 mm. diameter or nitrocellulose paper. Microtiter plates such as NUNC immunoplates, available from NUNC, Roskilde, Denmark, and nitrocellulose paper available from Schleicher and Schuell, Inc., as Item No. BA 83, are suitable examples. Other types of membranes as are known to those skilled in the art may be substituted for nitrocellulose paper, including aminobenzyl-oxymethyl (ABM) blotting paper, aminoethylthio-ether (APT) blotting paper, diethylaminoethyl (DEAE) blotting paper and zeta-probe charged modified nylon. See, Bio-Dot Microfiltration Apparatus Instruction Manual, Bio-Rad Labs, Richmond, California, pp. 1-30, 1984.

Approximately 0.06-0.12 ug of antibody [typically 100 ul of a 1 ug/ml solution] is coated onto each test vessel, i.e., microtiter well or nitrocellulose strip, preferably, 0.1 ug is used. The microtiter wells and beads are coated by known techniques, such as those

described in General ELISA Procedure, Dako, Corp., order no. 23/8710.

When nitrocellulose strips are used, approximately 100 ul of a 0.06-0.12 ug/ml antibody solution is applied to one side of each strip. Prior to applying the antibody to the strip, it is preferred to cover approximately 3/4 of the strip with a plastic material. The antibody solution is then applied to the exposed portion. The covered portion of the strip may be used as a handle without destroying the activity of the antibodies applied to the strip. It should be understood that the amount of antibody used as a coating on the solid phase may be varied depending on the totality of test conditions.

Human, goat, pig, donkey, horse, or cow immune antibodies to a retrovirus indicative of AIDS are useful in the method of the invention, although other immune antibodies reactive with human retroviral antigens may also be used. Presently, goat antibodies are preferred as the coating antibody. Monoclonal antibodies may be substituted for the immune polyclonal antibodies previously mentioned. Monoclonal antibodies are useful in a test designed to detect a single core or envelope protein or a specific combination of selected proteins.

Human antibodies to the AIDS retrovirus are obtained from the serum of infected individuals, either individual donors or pooled sera from multiple donors.

Pig, goat, donkey, horse and/or cow immune antibodies are obtained by immunizing an animal in accordance with known techniques. Typically, an animal is first vaccinated with from 5-500 ug, preferably 100 ug of a retroviral lysate mixed with Freund's complete adjuvant (1:1). Booster injections, begin 14-30 days after the first vaccination, and are administered approximately every 1-8 weeks, preferably every 2-4 weeks, and contain from 50-100 ug of viral lysate, with

or without Freund's adjuvant depending on the antibody titer measured semi-quantitatively by ELISA. Immunization continues until an antibody titer, measured by ELISA, of from 0.300-2.00 OD (measured by a Behring (ENI) ELISA) is detected. Once a sufficient antibody titer is measured, the serum is subjected to Western Blot analysis to confirm the presence of antibodies to both envelope and core proteins. It is preferred if the tested antibodies demonstrate the presence of antibodies directed to p 24 and gp 41, gp 120 and/or gp 160, as shown by Western Blot analysis. Once the aforementioned OD and Western Blot banding pattern are obtained, the animal is bled and its serum extracted.

The serum is partially purified, preferably according to the conventional saturated ammonium sulfate method, although other purification techniques such as polyethyleneglycol precipitation (R.J. Carter et al, J. Immunol. Methods, 26:213 (1979)) may be used. Briefly, saturated ammonium sulfate (up to 50% of the starting volume of serum) is used to precipitate the serum. After discarding the supernatant, the precipitate is redissolved to the original starting volume of the serum with 25% PBS-ethyleneglycol (vol/vol) and applied to a sieving gel according to the method described in K. Berg et al, Scand. J. Immunol., 11:489, 1930; K. Berg et al, J. Gen. Virol., 50:441, 1980. The fractions rich in IgG are determined using Partigen plates and collected. Partigen plates are available from Behring Diagnostics and the method is carried out in accordance with the manufacturer's instructions as described in package inserts. The IgG rich fractions should optimally contain 1mg/ml IgG, although fractions containing at least 0.6 mg/ml IgG are useful. If desired, total protein may be determined by the Lowry method. The IgG rich fractions which contain the desired antibodies to the retrovirus

are stored in a refrigerator or freezer until used. Preferably, 0.01% thimerosal is added as a preservative, although other preservatives known to those skilled in the art are also suitable.

Human sera, which as discussed hereinabove, may also be used as a source of the retroviral antibodies, are purified by the aforementioned saturated ammonium sulfate method.

Before use, the partially purified antibodies are tested by ELISA and Western Blot. It is believed that for purposes of the present method an ELISA OD of 0.300-2.000 OD and Western Blot banding pattern showing activity against both core and envelope proteins are required.

The retroviral antibodies obtained in the manner previously described are useful as both the antibody coated onto the solid phase, and for the formation of the aforementioned second immune complex. While it is preferred to use antibodies obtained from different sources for each purpose, the same antibodies may be used for both. That is, for example, when porcine immune antibody is used to coat the test vessel, it is preferred to use non-porcine antibodies for the formation of the second immune complex. However, when the same antibody is used, prior to formation of the second immune complex, it is necessary to neutralize any excess binding sites remaining on the coating antibody. It is believed that this procedure avoids undesirable binding of the anti-antibody required for formation of the third immune complex to the coating antibody, and therefore, preserves the integrity of the test system. The excess binding sites are generally neutralized by adding anti-antibodies against the coating antibody. Thus, for example, if the coating antibody is porcine IgG, the neutralizing antibodies are anti-porcine IgG.

Forming A Triple Sandwich Immune Complex

According to one embodiment of the present method, the retroviral antibodies are coated onto microtiter wells in accordance with known techniques. 0.06-0.12 ug, preferably, 0.1 ug (100 ul of a 1 ug/ml solution) of antibody is coated onto each well. Typically, 100 ul of a 0.1% BSA solution is then added to the wells to block non-specific binding, although other reagents as are known may be used, such as gelatine. Approximately 100 ul of a test sample, either body fluid or lysed cell material, is added to each well, and preferably incubated overnight (approximately 16 hours) at 4°C to thereby obtain a first immune complex comprising antibody-antigen.

It has been found that sufficient binding of antigen and antibody also occurs after incubation at 37°C for 2 hours. As can be appreciated, the 2 hour incubation period results in a significant reduction in total test time, an important consideration in emergency situations such as those often associated with organ transplantations.

After completion of the incubation period the liquid is discarded and the plates are washed 5 times, preferably with PBS-Tween, although other buffers may be used, such as Tris. No soaking time is required for the washes. Then, 100 ul of an approximately 1 ug/ml solution of retroviral antibodies are added to each well and incubated preferably for 1 hour at 37°C to thereby obtain a second immune complex comprising antibody-antigen-antibody. If desired, this incubation may be overnight at 4°C. Upon completion of the incubation, the liquid is discarded and the plates washed 5 times with buffer, as previously described. Then, from about 0.06-0.12 ug of anti-antibody, preferably, 100 ul of a 1 ug/ml solution of an anti-antibody, i.e., an antibody to the retroviral antibodies used to obtain the second immune complex, is added to each well and incubated for 1 hour at 37°C to

obtain a third immune complex comprising antibody-antigen-antibody-anti-body. The anti-antibodies, as previously mentioned, are against the retroviral antibodies used to obtain the second immune complex. Thus, if porcine IgG is used for this purpose, the anti-antibody is anti-porcine IgG. Rabbit or goat antiserum is preferred for this purpose. Such antisera are commercially available from Calbiochem and Dako, Corp. Typically, the commercially available anti-antibody preparations have an IgG concentration of from 0.6-1.2 mg/ml. The stock material is diluted accordingly, as previously described.

In one embodiment of the invention, the anti-antibody is labeled with a detectable atom such as a radioisotope or fluorescein, so that the triple sandwich immune complexes may be detected by radioimmunoassay or immunofluorescent assay techniques. If a radionuclide is used, I^{125} is preferred. In a preferred embodiment of the invention, the triple sandwich immune complex is detected by immunoenzymatic assay. In accordance with the immunoenzymatic assay, the anti-antibody is biotinylated, preferably with either biotin or biotin X, commercially available from Calbiochem. The biotinylation is accomplished by known techniques such as those described by in the Calbiochem Biochemical/Immunochemical Catalogue, 1987. It should be understood that any biotin derivative is considered useful.

According to this preferred embodiment, after incubation with the biotinylated anti-antibody, the plates are washed 5 times as previously described. Then, 100 ul of an avidin or strepavidin enzyme conjugate diluted as follows is added to each well and incubated for one hour at 37°C to allow binding of the enzyme conjugate to the biotinylated anti-antibody. A 1 mg/ml avidin of strepavidin enzyme conjugate solution

is diluted from 1:1000 - 1:5000, preferably, 1:1000. The avidin or strepavidin is enzyme conjugated, in accordance with known methods, to horseradish peroxidase, alkaline phosphatase, or Beta-D-galactosidase. It should be understood that other enzymes as are known to those skilled in the art are also useful. It is presently preferred to use avidin or strepavidin conjugated with horseradish peroxidase. Upon completion of the incubation of the enzyme and biotinylated third immune complex, the liquid is discarded and the plates washed 5 times as before. Then 100 ul of an appropriate enzyme substrate specific for the enzyme conjugate used, diluted accordingly, is added and incubated for 10-20 minutes at room temperature in the dark. Typically, the microtiter plate or other vessel, such as tube is covered with aluminum foil. The reaction of enzyme with an appropriate substrate is a color producing reaction. The substrate orthophenyldiamine (OPD) is preferred when horseradish peroxidase is used, although DAP, 3,3'-diaminobenzidine tetrahydrochloride dihydrate as well as other known substrates specific for horseradish peroxidase may be substituted. When alkaline phosphate is used, p-nitrophenylphosphate (BioRad, Catalogue no. M3358) is one suitable substrate.

OPD is commercially available as tablets from Electro Nucleonics Inc. and is prepared in accordance with the manufacturer's instructions prior to use (i.e., one tablet is dissolved in 5 ml of the substrate buffer supplied) and 100 ul of the prepared solution is added. After completion of the incubation period, the color producing reaction is stopped by addition of 50 ul of 2N H_2SO_4. Other reagents may be used to stop the reaction such as H_2O.

The optical density of the liquid contained in the wells is measured in a spectrophotometer at 492 nm

(reference 650 nm) as an indication of the presence or absence of antigens to a retrovirus associated with AIDS. The Behring ELISA Processor II is specifically designed for the purpose, but other instruments are also suitable.

A test sample is considered positive, i.e., containing retroviral antigens, if on an ELISA system the optical density of a sample is greater than the cut off point calculated by the formula $[\bar{X}(PC)+\bar{X}(NC)]/3$ = cutoff; X indicates the mean value of two readings. For example, if the mean value for the positive control (PC) is 0.450, and the mean value for the negative control (NC) is 0.106, the cut off point is calculated as 0.185, i.e., 0.450+0.106/3 = 0.556/3 = 0.185.

When the assay of the invention is carried out using nitrocellulose strips, the results of the test are evaluated by visually comparing the color developed by the positive and negative controls to the color produced by the test sample.

It should be understood that the amounts of reagents and incubation periods described may be varied depending on the totality of test conditions.

Diagnostic Test Kits

In another preferred embodiment of the invention, a diagnostic test kit for the detection of the presence or absence of antigens of a retrovirus indicative of AIDS, which bind with retroviral antibodies is provided; the test kit comprises retroviral antibodies, retroviral anti-antibodies, and means for detecting the formation of triple sandwich immune complexes between the antibodies, antigen and anti-antibodies. The means are present in an amount sufficient to perform said detection. The presence of the triple sandwich immune complexes may be detected by radioimmunoassay, immuno-enzymatic and immunofluorescent techniques, preferably, immuno-enzymatic means.

A preferred test kit includes nitrocellulose test strips, a set of tubes containing prediluted positive

and negative control references, a reagent control, and at least one sample tube containing a predetermined volume of buffer to which a predetermined amount of test sample is added. The reagent control is provided to assure that the reagents are functional. The nitrocelluose strips are coated on one side with from 0.06-0.12 ug of antibodies to a retrovirus indicative of AIDS. It is preferred that the strips are about 3/4 covered with plastic as previously described. The preferred kit also includes a second antibody to the AIDS retrovirus from a source different from the coating antibody, a biotinylated anti-antibody against the second retroviral antibody, an enzyme substrate, color reaction stopping reagent, and washing buffer. Preferably, predeveloped positive and negative reference strips and reagent control strips are provided in the kit. The predeveloped strips are used to evaluate the test results by a visual comparison with the sample test strips after completion of a color reaction. The test is considered positive if a test sample produces a color comparable to that of the positive control.

As previously mentioned, in accordance with the present assay method, the presence of absence of antigens of a human retrovirus indicative of AIDS may be detected directly from body fluids and lysed cell materials. Leukocytes are of particular interest. Leukocytes are collectable from the buffy coat resulting from centrifugation of heparinzed blood in accordance with known techniques. The cells so collected are typically lysed with 2.5% saponin or 0.1 - 1% Nonidit (NP40, available from Sigma). Other known lysing techniques are also considered useful.

In accordance with the assay methods described herein, a retroviral lysate (of a virus indicative of AIDS) is useful as a positive control. The viral

lysate is typically an inactivated, partially purified viral lysate which on testing in accordance with the method described herein gives an OD reading of between 0.250-1.100 at 492 nm (with a 650 nm reference), using OPD as the enzyme substrate. The viral lysate may be purified by known techniques, and inactivated by the known psoralen-UV irradiation method or heat (56°C for one hour). A partially purified, inactivated HIV-1 lysate is commercially available from Protatek International, Inc., St. Paul, Minnesota.

The following example illustrates the nature of the present invention, although it is understood that the invention is not limited thereto.

Example 1

A NUNC flat bottomed 96 well immuno plate was coated with goat anti-HIV-1 IgG obtained partially purified from Protatek International, Inc. The titer of goat IgG was measured to be approximately 1.0 mg/ml as determined by the Mancini technique against rabbit affinity purified anti-goat IgG (Mancini, G. et al, Immunochem. 2:235, 1965). The IgG was also tested by Quick Western Blot Assay and it was determined that antibodies to p24 and gp41 were present. The reference rabbit anti-goat IgG was obtained from Calbiochem, Catalog 401511. The goat antibody was diluted 1:1000 with PBS and 100 ul was added to each well of the immuno plate. After the diluted antibody was added to each well, the immuno plate was sealed with Parafilm and incubated overnight (16 to 18 hours) at 4°C, followed by washing 5 times with PBS on a Behring ELISA Processor II washing machine. After the final wash 0.1% (v/v) bovine serum albumin (BSA) diluted in PBS buffer was added to each well. The plate was then incubated for one hour at room temperature followed by washing 5 times as previously described. The assay was carried out in the following manner. An HIV-1 lysate, obtained from Protatek International, Inc. was diluted

with PBS, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000. 100 ul of the various dilutions were added to the wells at 4°C overnight. Serum samples from HIV-1 positive and negative individuals were used as positive and negative controls, respectively, and 100 ul at dilutions of 1:100 and 1:10 were added to the wells of the immuno plate. The immuno plate was covered with Parafilm and incubated overnight at 4°C. Upon completion of the incubation period the plate was washed five times with PBS - Tween, as previously described. Then, 100 ul of an approximately 1 mg/ml solution of human anti-HIV-1 IgG (in PBS) was added to each well. The human IgG was tested prior to use and demonstrated the presence of HIV core and envelope antibodies. Using NOR PARTIGEN plates it was determined that the Human IgG had an IgG concentration of about 1 mg/ml. (The PARTIGEN plates were obtained from Calbiochem Catalog No. 91001 and the IgG concentration was determined using the method described by the manufacturer.) The plates were incubated at 37°C for 1 hour followed by washing 5 times, as previously described. Then 100 ul of biotinylated goat antihuman IgG antiserum obtained from Calbiochem Catalog No. 401443, diluted 1:1,000 in PBS was added to each well and incubated at 37°C for 1 hour followed by 5 washings as previously described. Avidin conjugated horseradish peroxidase (obtained from Calbiochem Catalog No. 189728) diluted 1:1000 was added to the wells, and incubated for 1 hour at 37°C, followed by 5 washings at previously described. OPD tablets were dissolved in accordance with the manufacturers' instructions, and 100 ul of the resulting solution was added to each well and incubated between 10 and 20 minutes in the dark at room temperature to thereby obtain color. The reaction was stopped upon addition of 50 ul of 2N sulfuric acid (H_2SO_4). Results were determined by reading the plate on a Behring ELISA Processor II.

CLAIMS

1. An in vitro diagnostic method for the detection of the presence or absence of antigens of a human retrovirus indicative of Acquired Immune Deficiency Syndrome (AIDS) and/or AIDS related complexes (ARC) which bind to antibodies against said human retrovirus, which method comprises:

(a) contacting antibodies to the retrovirus with a biological fluid or cell lysate and incubating for a period of time and under conditions sufficient for the retroviral antigens in the biological fluid and the antibodies to form a first immune complex comprising antibody-antigen;

(b) contacting the first immune complex with antibody against the human retrovirus and incubating for a time and under such conditions sufficient for the retroviral antibodies and first immune complex to form a second immune complex comprising antibody-antigen-antibody;

(c) contacting the second immune complex with a retroviral anti-antibody against the antibody of step (b) and incubating for a period of time and under conditions sufficient for the second immune complex and anti-antibody to form a triple sandwich immune complex comprising antibody-antigen-antibody-anti-antibody; and

(d) detecting the triple sandwich third immune complex.

2. The method of claim 1, wherein the third immune complex is detected by immunological assay methods selected from the group consisting of a radioimmunoassay, immuno-enzymatic assay, and immuno-fluorescence assay.

3. The method of claim 2, wherein the immuno-enzymatic assay is an ELISA (enzyme-linked immunosorbance assay).

4. The method of claim 1, wherein the anti-antibody is biotinylated with biotin or biotin-X.

5. The method of claim 4, wherein the anti-antibody is biotinylated and further comprising the steps of:

(a) incubating the third immune complex with an enzyme conjugated avidin or strepavidin for a time under conditions sufficient for the enzyme conjugate to bind with the biotinylated anti-antibody;

(b) incubating the enzyme-conjugated third immune complex with an enzyme substrate specific for the enzyme of step (a), thereby producing color;

(c) stopping the color producing reaction of step (b); and

(d) evaluating the amount of color produced as an indication of the presence or absence of antigens of the human retrovirus.

6. The method of claim 1, wherein the antibody of step (a) is coated onto a solid phase selected from the group consisting of microtiter plates, nitrocellulose strips, glass beads, and plastic beads.

7. The method of claim 1, wherein the human retrovirus is HIV-1 or HIV-2.

8. The method of claim 1, wherein the biological fluid is human sera.

9. The method of claim 1, wherein the cell lysates are obtained from human leukocytes.

10. The method of claim 5, wherein the enzyme is selected from the group consisting of horseradish peroxidase, alkaline phosphatase and Beta-D-galactosidase.

11. A diagnostic test kit for the <u>in vitro</u>

antigen-antibody-anti-antibody, the means being present in an amount sufficient to perform said detection.

12. The diagnostic test kit of claim 11, wherein the means for detecting the formation of the immune complexes are immunological assay methods selected from the group consisting of radioimmunoassay, immuno-enzymatic and immunofluorescent assay means.

13. The diagnostic test kit of claim 11, wherein the nitrocellulose strips are coated with from 0.06-0.12 ug of a retroviral antibody preparation.

14. The diagnostic test kit of claim 11, wherein the anti-antibody is biotinylated with biotin or biotin-X

15. A diagnostic test kit for the in vitro detection of the presence or absence of antigens of a human retrovirus indicative of acquired immune deficiency syndrome (AIDS) or AIDS related complexes (ARC) by the formation of a triple sandwich immune complex comprising:

(a) a set of control tubes comprising positive and negative references;

(b) at least one reagent control tube;

(c) at least one sample tube containing a predetermined volume of buffer for dilution of test samples;

(d) a least one nitrocellulose strip for each tube, the nitrocellulose strips being coated with antibody to said retrovirus;

(e) prediluted antibody to said retrovirus;

(f) a biotinylated retroviral anti-antibody;

(g) avidin or strepavidin conjugated enzyme;

(h) enzyme substrate specific for the enzyme conjugate;

(i) a color producing reaction terminating reagent; and

(j) predeveloped positive, negative and reagent control strips for evaluating the results of the test by visually comparing the predeveloped strips with test strips after completion of a color change reaction.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/01026

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) C12Q 1/70; G01N 33/545; B65D 69/00
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II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [5]	Classification Symbols
U.S. Cl	435/5,7,805,810 436/531,808,809,810,811

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category * [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,E Y,E	US, A, 4,748,110 (D.A. PAUL) 31 May 1988 (31.05.88), See column 2, lines 7-36	1-15 7
Y	Journal of Immunological Methods, Volume 76, no. 1, issued 1985, J.S. McDOUGAL ET AL, "Immunoassay for the Detection and Quantitation of Infectious Human Retrovirus, Lymphadenopathy-Associated Virus (LAV)", pages 171-183 see pages 174 and 182.	1-15
Y	US, A, 4,034,074 (L.E.M. MILES) 05 July 1977 (05.07.77), See column 4, lines 1-22.	1-15

* Special categories of cited documents: [19]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 JUNE 1988	25 JUL 1988
International Searching Authority [1]	**Signature of Authorized Officer** [20]

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC ND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
Y	Science, Volume 233, issued 1986 18 July, F. CLAVEL ET AL, "Isolation of a New Human Retrovirus from West African Patients with AIDS" pages 343-346, see page 346.	7
A	US, A, 4,520,113 (R.C. GALLO ET AL) 28 May 1985 (28.05.85).	